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Studies on the association of EBV with Breast cancer have had notably inconsistent results. This is attributable partly to two persistent methodological problems: the technical challenges of localizing EBV to tumor cells, and the failure to address the epidemiological perspective, which could contribute to variability in EBV prevalence across studies. Toward this end, we have collected more breast cancer samples from China and India to investigate geographic differences. We also performed EBV analysis on microdissected tumor cells from PCR positive, EBER ISH positive and PCR positive, EBER negative samples. Our data showed there is no significant difference between conventional PCR and real-time PCR when targeting EBNA1 sequence on whole section samples. There is also no significant difference in the proportion of positive samples in different geographic regions. When amplifying DNA from microdissected tumor cells, the rate of detection of EBV was lower than by whole section PCR alone, but much higher than by EBER ISH. The discrepancy between EBER ISH and PCR in microdissected tumor cells requires further investigation.

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INTRODUCTION

With more conflicted reports on the association of EBV with breast cancer (1-6), choosing more methodologies and including samples from different geographical areas with varying prevalence of EBV associated malignancies become more important (7). Several commonly used methods, such as PCR, Southern blot hybridization and immunohistochemistry all have limitations in this study (see the annual report –2003 for this award). During last year, after we have collected samples from China and India, we concentrated on two approaches to study these samples in accordance with the recommendation by National Cancer Institute: real-time quantitative PCR, which allows measurement of the amount of viral DNA in archival tissue samples, and laser capture microdissection to improve localization of viral DNA to benign or malignant components of a tissue sample (8). This combined approach greatly increased sensitivity and specificity of the study, but it was still difficult to exclude occasional contamination of tumor cells by infiltrating lymphocytes or the presence of free virus in the tissue. In situ hybridization techniques such as EBER ISH may overcome this problem, however, our data and other reports showed only focal expression of EBER in tumor cells in rare cases and absence of EBER positive tumor cells in most PCR positive samples. All these obstacles made the study of the association of EBV with breast cancer very challenging. We need to be extremely careful when setting up assays and in interpreting data, to avoid erroneous conclusions.

BODY

All 79 breast cancer samples from China and 100 cases from India were screened for the presence of EBV genome by conventional PCR and real-time quantitative PCR using primers flanking the EBV nuclear antigen 1 (EBNA-1) region. EBER ISH was performed on positive cases to determine whether EBV expression occurs in the tumor compartment or in the infiltrating lymphocytes. PCR for defective heterogeneous (Het) EBV was also carried out on these cases to detect EBER negative cases harboring the defective EBV genome (9). (For the information of DNA isolation and EBER ISH and primer sequences used in conventional PCR and real time PCR, see Annual report-2002 for this award).

Table 1. Summary of results on 79 Chinese cases

EBV positivity on PCR for EBNA1 or for het EBV			EBER ISH on PCR positive cases
Conventional PCR	Real-time quantitative PCR	Conventional PCR for het EBV	
57% (45/79)	50% (39/79)	2.5% (2/79)	58% (26/45) Limited to lymphocytes, including 7 cases that had both lymphocytes and endothelial cells staining

The study results of 79 Chinese cases are summarized in Table 1. (Due to the delay of the arrival of Indian samples, we haven't finished the entire project yet. The results on Indian samples will be summarized in the final report). There is no significant difference between conventional PCR and real-time PCR results. The EBV loads were very low in most cases except two cases which are under further investigation. The data also suggest het EBV may not be a major form of EBV present in breast cancer tissues. EBER ISH showed some staining in 26 out of 45 PCR positive cases, but the reactive cells were all infiltrating lymphocytes, including 7 cases with staining of endothelial cells (one example is shown in Figure 1.). Our findings suggest that EBV is not present in breast cancer cells or all breast tumors do not express detectable levels of EBERs, even in the presence of EBV genome. More sensitive and specific methods, such as PCR on microdissected tumor cells may need to be used in this study. The positive staining of some endothelial cells is an interesting finding since no previous report has been made. This suggests EBV can infect a broader spectrum of cell types than previously suggested.

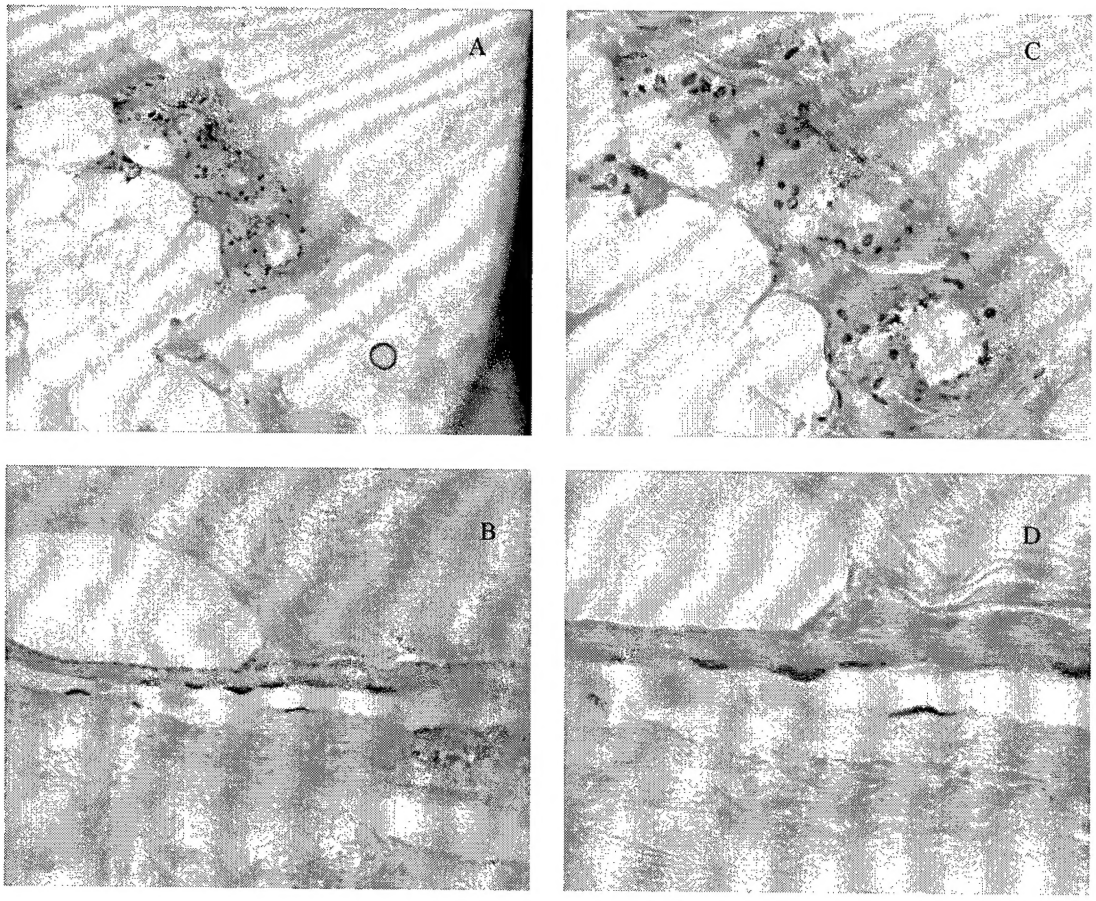

Figure 1. EBER ISH detected positive staining (dark blue cells) in some normal glandular cells in Panel A (low magnification) and Panel C (same section shown in higher magnification) in one case, some endothelial cells in Panel B (low power) and Panel D (high power) in another case.

We selected two groups of samples for microdissection study: samples that were PCR positive and EBER ISH positive in tumor or/and lymphocytes vs PCR positive but EBER negative samples. Positive tumor cells or infiltrating lymphocytes were microdissected from the EBER ISH sections using the Leica Laser Dissection System (Leica, Germany) in the first group. Tumor cells and lymphocytes randomly dissected from the H&E stained sections for the second group. Non-cellular fibrous tissue were also dissected as controls. DNA was isolated from microdissected cells (usually around 100 cells of each cell type were dissected from each sample). Usually one fourth of DNA was used to check DNA quality by amplifying the β-globin gene which generates two PCR products with lengths of 120 bp and 260 bp. The microdissected samples with amplifiable DNA underwent PCR of EBNA1. UNG system was incorporated in both PCR reactions to eliminate carry over contaminations. Both PCR reactions were amplified for 40 cycles.

The results of two representative samples are shown in Figure 2.

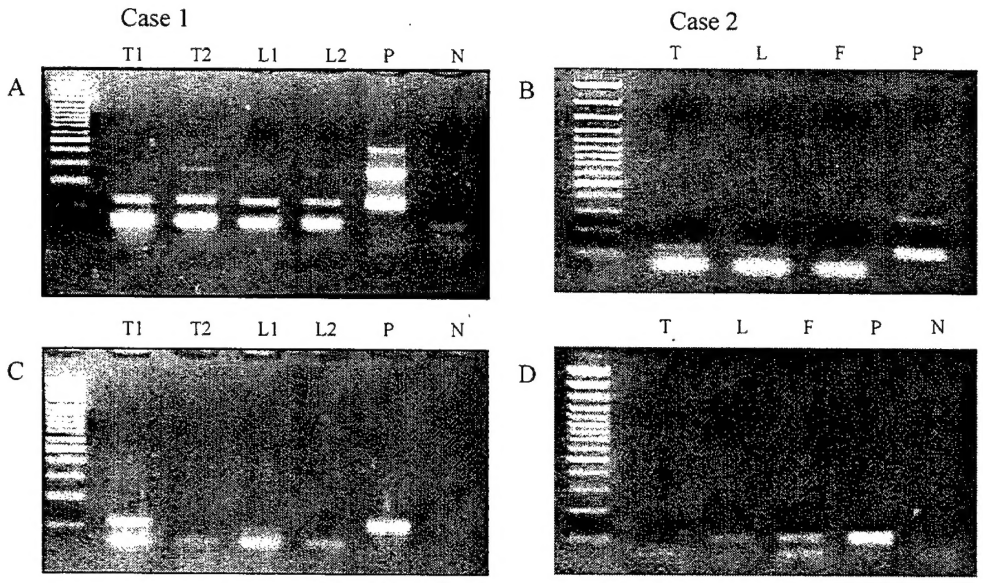

Figure 2. PCR on microdissected tumor cells (T), lymphocytes (L) and fibrous tissue (F) from two cases. Panel A & B are PCR products of β-globin showing there are amplifiable DNA in both cases. Panel C shows that only microdissected tumor cells are positive for EBNA1in Case 1. Panel D shows microdissected lymphocytes and non-cellular fibrous control are positive in case 2. First lane in each panel is 100 bp DNA marker. P – Positive control of a B cell line harboring EBV genome. N- No template control.

Five cases from group1 and 8 cases from group 2 had amplifiable DNA for the study of the presence of EBV by amplifying the EBNA1 region. The results from group1 and group2 samples are summarized in Table 2. and 3, respectively.

Table 2. EBV positivity by PCR on microdissected cells from EBER ISH slides.

Case ID	Tumor cells	Lymphocytes
US39[1]	+	-
US38[2]	-	-
US63[2]	+	+
US68[2]	-	+
CN15[2]	-	+

1 – Half tumor cells and some infiltrating lymphocytes were stained positive for EBER ISH in this case. 2 – Only few infiltrating lymphocytes were positive for EBER ISH in

these cases. Tumor cells were randomly microdissected from these cases.

Table 3. EBV positivity by on microdissected cells from H&E slides.

Case ID	Tumor cells	Lymphocytes	Fibrous tissue
US 1	+	-	NA
US 2	-	+	NA
US3	-	-	NA
US4	-	+	NA
CN1	+	-	-
CN2	-	+	+
CN3	+	+	-
CN4	+	-	NA

PCR amplifying EBNA1 on microdissected cells demonstrated high EBV positive rate (2/5 on group1 samples and 4/8 on group 2 samples) on tumor cells irrespective of EBER ISH status. Although the case number is small, the initial data suggest the "gold standard" EBER ISH for EBV associated malignancies may not reveal all EBV infections of breast carcinoma. However, while the PCR on the microdissected cells showed intriguing data, one of three non-cellular fibrous controls also showed positive result, which causes concerns about the interpretation of EBV infection without EBER expression. More cases need to be studied with extreme negative controls.

KEY RESEARCH ACCOMPLISHMENTS

- Established EBER in situ hybridization for localization of EBV infected cells.
- Assayed 100 USA cases, 79 China cases, 23 Saudi Arabia cases and 40 of Indian cases of breast carcinoma for EBV by PCR and EBER ISH.
- Developed Real-Time PCR assay for the EBNA1 region and also an assay for the defective EBV.
- Organized a data- base for the experimental findings.
- Developed LMD techniques for isolation of tumor cells and other cellular and non-cellular compartments in tissue sections.

REPORTBLE OUTCOMES:

An abstract titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.

One technologist was trained for molecular biology and Laser microdissetion techniques supported by this reward.

A couple of manuscripts are preparing based on the study of this award.

CONCLUSIONS:

Our study showed EBV positivity rate is around 40-50% by PCR on whole tissue sections for EBNA1. The positive rate is similar for different geographic areas. PCR on microdissected tumor cells also detected virus in 40-50% of specimens of PCR positive samples irrespective of EBER status. The data suggest EBER ISH may be not the definitive marker for EBV in breast carcinoma. However, the specificity of the PCR for intracellular virus needs to be confirmed by additional studies. Rarely normal glandular cells and quite often, endothelial cells were found to be positive besides infiltrating lymphocytes suggesting EBV may infect more cell types than previously suggested. The origin of EBV in breast tumor cells most likely come from infected infiltrating lymphocytes through cell to cell contact. Although it is possible that the neoplastic cells arise from normal breast glands that harbor EBV, it is uncommon to find infection of normal glandular tissue and EBERs is rarely demonstrated in a high proportion of tumor cells.

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APPENDICES

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